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The IGF-1/IGF-1R signaling axis in the skin: a new role for the dermis in aging-associated skin cancer

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Abstract

The appropriate response of human keratinocytes to UVB is dependent on the activation status of the IGF-1 receptor. Keratinocytes grown in conditions where the IGF-1 receptor is inactive, inappropriately replicate in the presence of UVB-induced DNA damage. In human skin epidermal keratinocytes do not express IGF-1, so the IGF-1 receptor on keratinocytes is activated by IGF-1 secreted from dermal fibroblasts. We now demonstrate that the IGF-1 produced by human fibroblasts is essential for the appropriate UVB response of keratinocytes. Furthermore, the expression of IGF-1 is silenced in senescent fibroblasts in vitro. Using quantitative RT-PCR and immunohistochemisty, we can demonstrate that IGF-1 expression is also silenced in geriatric dermis in vivo. The diminished IGF-1 expression in geriatric skin correlates with an inappropriate UVB response in geriatric volunteers. Finally, the appropriate UVB response is restored in geriatric skin in vivo via pretreatment with exogenous IGF-1. These studies provide further evidence for a role of the IGF-1R in suppressing UVB-induced carcinogenesis, suggest that fibroblasts play a critical role in maintaining appropriate activation of the keratinocyte IGF-1R, and imply that reduced expression of IGF-1 in geriatric skin could be an important component in the development of aging-related non-melanoma skin cancer.

Keywords

Aging; IGF-1R; skin cancer; keratinocytes; fibroblasts; UVB

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Introduction

Conclusive evidence has demonstrated that the major environmental risk factor for non-melanoma skin cancer is exposure to the UVB wavelengths found in sunlight. Although skin cancer can occur at any age, there is a strong correlation between the development of skin cancer and advancing age as evidenced by the fact that a majority of skin malignancies are found in people over the age of 60 years [Kraemer, 1997]. Childhood exposure to UVB is believed to be one of the most critical risk factors in the development of skin cancers in adults [Whiteman *et al*., 2001]. This explanation proposes that epidermal keratinocytes acquire UVB-induced tumorigenic mutations in childhood which accumulate over time in selected populations of cells that manifest as skin cancers later in adult life [Kraemer, 1997; Shelton *et al*., 1999; Krtolica *et al*., 2001; Parrinello *et al*., 2005]. However, we have recently proposed a distinct model to explain the association of aging with the development of skin cancer based on the role of the IGF-1 receptor (IGF-1R) on the response of human keratinocytes in vitro to UVB exposure [Lewis *et al*., 2008a]. We define the normal appropriate response of skin to UVB irradiation as one where keratinocytes do not permit replication of UVB-induced DNA damage to daughter cells. Therefore, to attain this appropriate UVB response, keratinocytes will repair UVB-induced DNA damage, become senescent, or following extreme UVB exposure undergo apoptosis. During an appropriate UVB response, one should not observe cellular replication proteins and UVB-induced DNA damage within the same keratinocyte. Keratinocytes that are replicating unrepaired UVB-damaged DNA could establish permanent chromosomal mutations that lead to initiated carcinogenic cells. We now demonstrate that UVB-irradiation of geriatric skin leads to the creation of keratinocytes that have the potential to become initiated carcinogenic cells. This inappropriate UVB response is not observed following UVB irradiation of young adult skin. Furthermore, the role of IGF-1R activation in the appropriate UVB response is demonstrated by the prevention of the inappropriate UVB response in geriatric skin by pretreatment with IGF-1.

Historically, the dogma regarding the initiation of non-melanoma skin cancer has been that DNA damage inflicted on individuals from 3-17 years old resulted in “initiated” keratinocytes that required an additional 30-40 years to result in the development of NMSC tumors. Our data indicates that this path to carcinogenesis is not the only one (or in fact may not be even the predominant one). We demonstrate that UVB-induced initiated keratinocytes occur commonly in geriatric skin but not in young adult skin; a process that is dependent on the activation status of the IGF-1R on epidermal keratinocytes. We demonstrate that the silencing of IGF-1 expression in geriatric dermis results in the enhanced development of initiated keratinocytes in geriatric skin. These data and the accompanying new paradigm of how carcinogenesis is initiated in geriatric skin represents a major departure from previous theories on non-melanoma skin carcinogenesis as well as presenting new and interesting therapeutic interventions.

Results

Exogenous IGF-1 alters the response of normal human keratinocytes to UVB irradiation

How keratinocytes in the epidermis respond to UVB exposure is critically important in determining the probability of UVB-irradiated keratinocytes evading the formation of cancer-inducing DNA mutations. We have previously reported that the activation status of the IGF-1R is a critical mediator of UVB-induced apoptosis in vitro [Kuhn *et al*., 1999; Lewis and Spandau, 2007; Lewis *et al*., 2008b]. The sensitivity of the IGF-1R in this response was examined by titrating the concentration of IGF-1 added to EpiLife NoIn media (from 5 ng/ml to 25 ng/ml IGF-1) to determine the lowest protective concentration of IGF-1. As a control for the specificity of IGF-1, keratinocytes were treated with EpiLife NoIn media containing 25 ng/ml of insulin. Following UVB irradiation, the sensitivity to UVB-induced apoptosis was assayed by measuring the caspase 3 specific activity in keratinocyte cell lysates. No protection from UVB-induced apoptosis was found in insulin-treated keratinocytes (Figure 1A), confirming that the observed resistance to UVB-induced apoptosis is specific to the IGF-1R. In contrast, concentrations as low as 5 ng/ml IGF-1 offered substantial protection from UVB-induced apoptosis (Figure 1A). Irradiation of keratinocytes with lower doses of UVB demonstrated that IGF-1R activation was required for UVB-dependent stress-induced senescence (Figure 1B). UVB-induced keratinocyte senescence protects keratinocytes from propagating UVB-induced mutations. Therefore, during an appropriate UVB response markers of cellular DNA replication will not be observed in keratinocytes that have UVB-induced DNA damage. UVB-irradiation of keratinocytes with an activated IGF-1R results in two populations of cells: one population that consists of non-mitotic cells that contain unrepaired DNA damage (appearing green) and a second population that replicating but contain no DNA damage (appearing red, Figure 1C, panel *vi*). In contrast, keratinocytes that lack IGF-1R activation exposed to UVB will contain proliferating keratinocytes with DNA damage (appearing yellow, Figure 1C, panel *v*).

The keratinocyte UVB-response in vitro is altered by IGF-1 secreted by fibroblasts

Proper function of the skin requires the synergistic interactions between dermal fibroblasts and epidermal keratinocytes. The proliferation, differentiation, and stress responses of keratinocytes are greatly altered when deficiencies in dermal fibroblasts occur. We hypothesize that the normal UVB-response (defined as protection from carcinogenic initiation) of keratinocytes is also highly dependent on the status of dermal fibroblasts. As described above, we have defined conditions where the appropriate UVB-response of normal human keratinocytes grown in tissue culture can be altered by differential activation of the IGF-1R. We believe these culture conditions that alter the UVB-response in keratinocytes in vitro reflect instances of deficient fibroblast function in vivo. To determine if normal human fibroblasts can produce a protective factor which can activate the IGF-1R and restore the normal UVB response of keratinocytes, normal human fibroblasts were grown in the EpiLife NoIn media for forty-eight hours (referred to as Conditioned Media, NoIn-CM) and then the NoIn-CM was removed from the fibroblasts and filter sterilized. Keratinocytes were grown in EpiLife NoIn media for 48 hours and subsequently the media was replaced with EpiLife Complete media, with NoIn media, or NoIn-CM from fibroblasts.

One hour following media exchange, the keratinocytes were irradiated with 0 or 400 J/m^2 of UVB and subsequently assayed for the induction of apoptosis. As seen in Figure 2A, UVB irradiation induced a modest induction of apoptosis in keratinocytes treated with EpiLife Complete media and substantial apoptosis in keratinocytes grown in NoIn media. However, keratinocytes treated with NoIn-CM were resistant to UVB-induced apoptosis, similar to those treated with EpiLife Complete media. These data indicate that normal human fibroblasts produced a diffusible factor into the EpiLife insulin-deficient media that protected the keratinocytes from UVB-induced apoptosis. To determine if the protective factor produced by fibroblasts was IGF-1, we added a neutralizing antibody specific to IGF-1 to the fibroblast NoIn-CM. Addition of the IGF-1 neutralizing antibody prevented complete protection of UVB-induced apoptosis (Figure 2A). As expected, the addition of the neutralizing IGF-1 antibody to EpiLife Complete media did not alter the keratinocyte UVB response indicating a lack of cross-reactivity to the insulin in the EpiLife Complete media by the anti-IGF-1 antibody. At the concentration of IGF-1 antibody used, the manufacturers (Calbiochem, α-IGF-1 Ab-2) state that 50% of the IGF-1 will be inhibited corresponding to the approximate level of inhibition observed in our assays.

The expression of IGF-1 in fibroblast cultures can be suppressed by the transfection of IGF-1-specific siRNA. Within twelve hours following transfection with IGF-1 siRNA, the expression of IGF-1 was suppressed by over 80% (Figure 2B). To determine if the inhibition of IGF-1 expression in fibroblasts also abolishes the protective effect on keratinocytes found in fibroblast-conditioned media, fibroblasts were transfected with nonsense or IGF-1-specific siRNA. Twenty-four hours following transfection, the media on the fibroblasts was replaced with EpiLife NoIn media for an additional forty-eight hours. At that time, the conditioned media was collected and the cells harvested. Correspondingly, conditioned media from fibroblasts transfected with nonsense siRNA protected keratinocytes from UVB-induced apoptosis (Figure 2C). However, conditioned media taken from fibroblasts transfected with IGF-1 siRNA failed to protect keratinocytes from UVB-induced apoptosis illustrating the importance of fibroblast-derived IGF-1 in regulating the response of keratinocytes to UVB irradiation (Figure 2C).

Senescent fibroblasts silence IGF-1 expression and fail to induce the appropriate UVB-response in keratinocytes

It has been previously reported that IGF-1 expression was lost once diploid fibroblast cell lines reached replicative senescence [Ferber *et al*., 1993]. We wanted to determine if normal human fibroblasts directly isolated from skin also silenced IGF-1 expression following the induction of senescence. Therefore, we grew normal human fibroblasts until they became replicatively senescent or induced premature senescence in fibroblasts via oxidative stress. Replicatively senescent fibroblasts were obtained following 147 population doublings, (our definition of senescence is lack of proliferation over a period of 14 days). To determine if stress-induced senescence in fibroblasts silenced IGF-1 expression, fibroblasts were treated with 600 μM hydrogen peroxide (H_2O_2) for two hours and then maintained in culture for an additional 72 hours. The senescent phenotype of each of these fibroblasts was confirmed by staining the cultures for senescence- associated β-galactosidase activity (Figure 3A). Analysis of IGF-1 mRNA expression revealed significant silencing of IGF-1 expression

equally in both stress-induced and replicatively senescent fibroblasts (Figure 3B). Analysis of cell lysates from replicating and stress-induced senescent fibroblasts confirmed the silencing of IGF-1 expression at the protein level and also demonstrated increased expression of the senescence-associated protein p21 (Figure 3C). Furthermore, conditioned EpiLife NoIn media derived from each of the senescent fibroblast cultures failed to protect keratinocytes from UVB-induced apoptosis, ostensibly due to the observed silencing of IGF-1 expression in the senescent fibroblasts (Figure 3D). These data verify the silencing of IGF-1 expression in senescent fibroblasts and identify potential consequences to keratinocytes interacting with these senescent fibroblasts.

Silencing of IGF-1 expression in aging human dermis

If aging fibroblasts in geriatric dermis silence IGF-1 expression as senescent fibroblasts in vitro do, the UVB response of geriatric keratinocytes could be altered and lead to an increased carcinogenic potential. Because the incidence of non-melanoma skin cancer rises sharply with advancing age, our hypothesis suggests that the inappropriate response of geriatric skin to UVB irradiation involves components of the IGF-1R signaling pathway. Therefore, differences in the expression pattern of these proteins in young adult skin versus aged skin should be detected. We have examined skin specimens obtained from sun-protected anatomic locations representing young adult (20-28 years old) and geriatric (≥65 years old) individuals. As seen in Figure 4A (panels *i* and *ii*), young adult skin stained with antibodies specific for IGF-1 identified dermal fibroblasts which stained intensely for IGF-1 expression (indicated by black arrows). In contrast, fibroblasts in sections derived from geriatric skin contained little to no detectable IGF-1 expression (white arrows; Figure 4A, panels *iii* and *iv*). While IGF-1 is not expressed by epidermal keratinocytes, secreted IGF-1 could also be found throughout the epidermis in young adult skin while the epidermis of geriatric skin again demonstrated reduced IGF-1 epidermal staining. Consistent with these immunohistochemical assays, quantitative RT-PCR analyses of skin derived from geriatric subjects contained significantly reduced levels of IGF-1 mRNA transcripts when compared to young adult skin (Figure 4B). Immunohistochemical staining of these specimens with IGF-1R antibodies did not reveal any differences between geriatric skin and young adult skin (data not shown). However, immunohistochemical analysis of young adult epidermis demonstrated high levels of staining using an antibody specific to activated (tyrosine-phosphorylated) IGF-1Rs on the cell membranes of keratinocytes (Figure 4C). Treatment of the slides with tyrosine phosphatases prior to immunohistochemical analysis eliminated the staining by this antibody (data not shown). This staining pattern was not seen in sections of geriatric skin (Figure 4C).

Inappropriate response of geriatric epidermal keratinocytes to UVB irradiation in vivo

In order to further investigate correlations between aging related non-melanoma skin cancer and aging-associated alterations in the IGF-1/IGF-1R signaling pathways, we have developed an assay to quantify the integrity of the normal keratinocyte UVB response in skin in vivo. The assay is based on in vitro studies that demonstrated the appropriate response of skin to UVB irradiation will prevent keratinocytes containing DNA damage from proliferating (Figure 1C). This model predicts that when fully-functional young skin is irradiated with UVB, no keratinocytes containing UVB-damaged DNA should be

proliferating (appropriate UVB response). However, if the normal UVB response is altered in aged skin, UVB-irradiated geriatric skin should possess some keratinocytes that are proliferating despite the presence of UVB-damaged DNA (inappropriate UVB response). Proliferating keratinocytes are identified using antibodies to Ki67 and UVB-induced DNA damage is detected using antibodies to thymidine dimers. Geriatric (≥65 years old) and young adult (20-28 years old) volunteers were irradiated with 350 J/m^2 of UVB on non-sun exposed skin and biopsies were obtained of the irradiated skin and non-irradiated control skin 24 hours post-irradiation. These biopsies were examined for basal layer keratinocytes that stained positive for Ki67, thymidine dimers, and for keratinocytes that stained positive for both Ki67 and thymidine dimers. Figure 5A provides an example of a dual-positive keratinocyte (Figure 5A, panels *i*, *ii*, and *iii*) and an example of a replicating cell that does not contain DNA damage (Figure 5A, panels *iv*, *v*, and *vi*). As seen in Fig. 5B, very few double-positive (Ki67 and thymidine dimer) could be detected in UVB-irradiated young adult skin. This result supports the idea that young adult skin has an appropriate response to UVB exposure. In contrast, UVB-irradiated geriatric skin yielded many replicating keratinocytes that contained UVB-induced DNA damage (Fig. 5B), our definition of an inappropriate UVB response. When only thymidine dimer positive keratinocytes were calculated, UVB-induced DNA damage was mostly repaired in young adult skin after 24 hours while geriatric skin still harbored many UVB-induced DNA lesions (Fig. 6A). In contrast, when only Ki67 positive keratinocytes were analyzed, the proportion of proliferative keratinocytes in the basal layer was the same in young and geriatric skin (Fig. 6B). In addition, the proliferative ratio of keratinocytes was not influenced by UVB irradiation in either young adult or geriatric skin (Fig. 6B). Thus, geriatric skin does respond inappropriately to UVB irradiation in that keratinocytes that have DNA damage retain the capacity to undergo cellular replication.

Treatment with IGF-1 restores appropriate UVB response to geriatric skin

We have demonstrated that IGF-1 expression is silenced in geriatric dermis resulting in a deficient activation of the IGF-1R in the overlying epidermal keratinocytes. The lack of IGF-1/IGF-1R signaling in geriatric skin correlates with an inappropriate UVB-response in aged skin. Therefore, experiments were conducted to determine whether supplementation of IGF-1 in aged skin can restore the youthful, appropriate UVB-response to geriatric skin. If small amounts of IGF-1 are injected intradermally immediately below the epidermis, activation of keratinocyte IGF-1R can be detected (Figure 7A, panel *ii*). The specificity of this activation is demonstrated because the topical treatment of the skin with a specific small molecule inhibitor of the IGF-1R (AG 538) prior to IGF-1 injection completely abrogates the effects of IGF-1 (Figure 7A, panel *iii*). Subsequently, three regions of sun-protected skin on the lower back of geriatric volunteers were isolated. One region of skin was injected with 50 μl of sterile saline and a separate area was intradermally injected with 50 μl of saline containing 200 ng of recombinant human IGF-1 (Tercica, Inc; Brisbane, CA). Thirty minutes following the intradermal injections, the saline injected site and the IGF-1 injected site was irradiated with a UVB dose of 350 J/m^2. Twenty-four hours post-irradiation, punch biopsies of control unirradiated skin and both the UVB-irradiated skin areas treated with saline or IGF-1 were removed and processed for the detection of Ki67 and thymidine dimers. Irradiation of saline-injected sites on geriatric volunteers yielded equivalent numbers

of dual-staining basal layer keratinocytes as previously described (compare geriatric skin results in Figures 5B and 7B). However, the IGF-1 treatment prior to UVB irradiation restored the appropriate UVB response in geriatric skin (Figure 7B).

Discussion

The functional integrity of the skin is critically dependent on the many synergistic interactions between fibroblasts in the dermis and epidermal keratinocytes. One such interaction is the secretion of IGF-1 by dermal fibroblasts which activates the IGF-1R on epidermal keratinocytes. Various lines of evidence indicate that the IGF-1 signaling pathway is crucially important for the appropriate response of keratinocytes to ultraviolet radiation (specifically UVB). This appropriate response of keratinocytes to UVB is important to suppress oncogenic transformation in the skin, as the main environmental risk factor for developing non-melanoma skin cancer is exposure to the UVB components of sunlight. The functional activation of the IGF-1R enhances the survival rate of human keratinocytes following UVB irradiation [Lewis *et al*., 2008a; Kuhn *et al*., 1999; Lewis and Spandau, 2007; Lewis *et al*., 2008b]. However, as a consequence of cell survival, these keratinocytes cannot continue cellular replication and they become senescent. If the IGF-1R is inactive at the time of UVB-irradiation, keratinocytes are less likely to survive and a large percentage of the keratinocytes will undergo apoptosis[7]. However, surviving keratinocytes do not undergo stress-induced senescence, do not repair UVB-damaged DNA, and can continue to proliferate with the potential of converting the UVB-damaged DNA into carcinogenic mutations. We have now demonstrated that normal human fibroblasts grown in vitro secrete IGF-1. The production of IGF-1 by fibroblasts is capable of altering the response of normal human keratinocytes to UVB irradiation. Furthermore, we have shown that normal fibroblasts directly isolated from human tissue fail to express IGF-1 when they become replicatively senescent, confirming the previously published reports using fibroblast cell lines. Additionally, fibroblasts induced to become senescent via oxidative stress or UVB exposure also have reduced IGF-1 expression and once again the reduction in IGF-1 expression is accompanied by the failure of fibroblast-conditioned medium to protect keratinocytes from UVB-induced apoptosis.

Although skin cancer can occur at any age, there is a strong correlation between the development of skin cancer and increasing age [Kraemer, 1997]. Eighty percent of all skin cancers are found in people over the age of 60; therefore, age is a risk factor for the development of skin cancer. While the correlation between aged epidermis and skin is apparent, the mechanism responsible for this relationship remains obscure. The historical explanation for the correlation between skin cancer and aging argues that UVB damage inflicted on skin during adolescence initiates mutations in keratinocytes that are selectively enriched over many decades until enough genetic changes have accumulated in these keratinocytes that they become carcinogenic. Recent data from a variety of labs have proposed a modification of this theory based on changes in the stromal support cells of epithelial cells with advancing age [Shelton *et al*., 1999; Krtolica *et al*., 2001; Parrienllo *et al*., 2005]. This hypothesis states that the selection of mutated cells is accelerated in aged tissue due to inflammatory changes that have been observed in senescent fibroblasts supporting epithelial cell growth [Shelton *et al*., 1999; Krtolica *et al*., 2001; Parrienllo *et al*.,

2005]. The integration of data from our lab on the role of the IGF-1R and normal UVB response of keratinocytes with data describing the declining production of IGF-1 by senescent fibroblasts have led to a third hypothesis correlating aging skin with the development of skin cancer (Figure 8). As noted previously, keratinocytes express the IGF-1R but they do not synthesize IGF-1 [Tavakkol *et al*., 1992]. Dermal fibroblasts support the proliferation of keratinocytes in the epidermis by secreting IGF-1. However, dermal fibroblasts in geriatric dermis produce far less IGF-1 than fibroblasts in young adult dermis, implying that keratinocytes in aged skin may be supplied with an insufficient concentration of IGF-1. We believe this decrease in IGF-1 expression with advancing age is a major component of the increase in non-melanoma skin cancer seen in geriatric patients. A corollary of this hypothesis would be that individuals with an increased activation of the IGF-1R might have some protection from UVB-induced skin cancer that could be detected by a decrease in skin cancer incidence. IGF-1 and insulin have very similar molecular structures and high concentrations of insulin will activate the IGF-1R. Patients with type II diabetes frequently have to take exogenous systemic insulin to overcome their insulin resistance. We hypothesized that the high systemic insulin levels in these patients would compensate for an aging-dependent decline in IGF-1 in the skin by inadvertently maintaining IGF-1R activation despite declining levels of IGF-1. If this were true, our control population would have an increasing incidence of skin cancer with advancing age, while the type 2 insulin-using diabetic patient cohort would not see a change in skin cancer incidence with age. In fact, the use of insulin was protective of the age-dependent increase in skin cancer incidence [Chuang *et al*., 2005].

It is important to distinguish between the role that IGF-1 plays in the *initiation* of UVB-induced skin cancer [Lewis *et al*., 2008a] and the previously well-documented activity that IGF-1 plays in *promoting* a variety of epithelial tumors [Pollak, 2008; Lann and LeRoith, 2008; Dziadziuszko *et al*., 2008]. In geriatric skin, diminished expression of IGF-1 in geriatric skin leads to uncharacteristically decreased activation of the IGF-1R in the epidermis. When keratinocytes are exposed to UVB in the absence of IGF-1R activation, the normal protective response to UVB is altered, so that keratinocytes with DNA damage fail to undergo stress-induced senescence and are capable of replicating chromosomes containing the UVB-damaged DNA. Therefore, the lack of IGF-1R activation at the time of UVB irradiation increases the probability of a cancer-*initiating* event. Previous reports of IGF-1 increasing carcinogenesis were in the context of *promoting* the growth of previously initiated cells, a distinctly different process. [Lewis *et al*., 2008a].

In summary, these studies provide further evidence for a role of the IGF-1R in suppressing UVB-induced carcinogenesis, suggest that fibroblasts play a critical role in maintaining appropriate activation of the keratinocyte IGF-1R, and imply that reduced expression of IGF-1 in geriatric skin could be an important component in the development of aging-related non-melanoma skin cancer.

Experimental procedures

Isolation and culture of fibroblasts and keratinocytes

Our protocol for the isolation and culture of keratinocytes has been previously described [Kuhn *et al*., 1999]. Briefly, excised foreskin tissue was washed with antibiotics, the tissue minced, and individual cells released from the tissue by trypsin digestion. Keratinocytes and fibroblasts were separated by differential resistance to treatment with EDTA. Isolated keratinocytes were grown in EpiLife Complete media (Cascade Biologics, Portland, OR) supplemented with human keratinocyte growth supplement (HKGS; Cascade Biologics) and 1000U Penicillin-Streptomycin (Roche). EpiLife NoIn media is identical to EpiLife Complete *except* it contains no insulin. All experiments were conducted using sub-confluent, low passage primary normal human keratinocytes. Fibroblasts were grown in Dulbecco's Modified Eagles medium containing 10% fetal calf serum. All relevant procedures using human tissue have been approved by the Indiana University School of Medicine Institutional Review Board.

UVB irradiation

UVB irradiation of normal human keratinocytes was accomplished using two Philips F20T12/UV sources as previously described [Cotton and Spandau, 1997]. The intensity of the UVB source was measured prior to each experiment using an IL1700 radiometer and a SED240 UVB detector (International Light, Newburyport, MA) at a distance of 8 cm from the UVB source to the monolayer of cells. Normal human keratinocytes were irradiated in EpiLife media (Cascade Biologics) and returned to standard incubation conditions (37°C and 5% CO_2). EpiLife medium absorbs all of the UVC wavelengths emanating from the light source without absorbing significant amounts of UVB wavelengths.

Senescence-associated β-galactosidase assays

Keratinocytes were washed twice with PBS and fixed with 2% formaldehyde/0.2% glutaraldehyde at room temperature for 10 minutes. After two additional washes with PBS, 2 ml of staining solution (150 mM sodium chloride, 25.2 mM sodium phosphate dibasic, 7.36 mM citric acid, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 1 ng/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside, pH 6.0) [Dimri *et al*., 1995], were added to the cells and they were incubated at 37°C overnight. The cells were again washed with PBS and photographed by bright field microscopy to count blue cells and phase contrast microscopy to count total cells. At least four fields (100X magnification, approximately 200-600 cells/field) were counted for each plate of cells; at least two plates of cells for each condition (or cell type) were assayed in each experiment.

siRNA transfection

Normal human fibroblasts were transfected with 1 nM IGF1-siRNA (Qiagen Gene Globe validated siRNA, #sI02664060) using HiPerfect transfection reagent (Qiagen #301705). Control transfections were performed identically using non-specific, scrambled siRNA (Control siRNA, Alexafluor 488, Qiagen Gene Globe #1022563).

Human UVB response assay

In accordance with the Declaration of Helsinki, written informed consent was obtained from all participants before enrollment in the study. All studies were approved by the Institutional Review Board at Indiana University Medical Center. Volunteers recruited for these experiments had Fitzpatrick Skin Types I or II (indicating fair-skinned individuals). Subjects were excluded from the study if they had a history of recent tanning bed use or therapeutic UV treatments, had a history of photosensitive skin disease, were taking medications which are photosensitizing, had allergies to xylocaine anesthesia, or had a history of abnormal scar formation. Subjects who met these criteria were separated into two groups; the first group consisted of individuals between the ages of 20 and 28 years old while the second group consisted of individuals greater than 65 years old. On Day 1 of the protocol, a 2 cm^2 area of the subject's lower back was isolated and irradiated with dose of 350 J/m^2 of UVB. In Fitzpatrick Skin Types I and II, this dose of UVB is sufficient to cause a minimal erythematous reaction. Twenty-four hours following UVB exposure, the irradiated skin, as well as unirradiated adjacent skin, were removed by punch biopsy, (a 4 mm punch biopsy of the UVB-treated area and a 3 mm punch biopsy of uninvolved skin on the same hip at least 10 cm away). Thin paraffin-embedded sections from unirradiated and UVB-irradiated biopsies were simultaneously stained with antibodies to Ki67 and thymidine dimers. Secondary antibodies that specifically detect only one of the primary antibodies are conjugated to the fluorescent dyes AlexaFluor 488 (detecting Ki67, emitting green wavelengths), and AlexaFluor 568 (detecting thymidine dimers, emitting red wavelengths). Images were captured sequentially along the entire length of the biopsy specimen (3mm non-irradiated, 4mm irradiated) using a Nikon Optiphot fluorescent microscope. These images were analyzed by counting the number of keratinocytes in contact with the basement membrane that are Ki67(+), thymidine dimer(+), and Ki67(+):thymidine dimer(+). These numbers were expressed as a percentage of total basal layer keratinocytes in the biopsy specimen (determined by counting basal layer keratinocytes for each specimen on H&E-stained slides).

Specific protocols for caspase 3 assays, quantitative reverse-transcription PCR, immunoblotting, immunohistochemistry, and immunofluorescence can be found in the Supplemental Material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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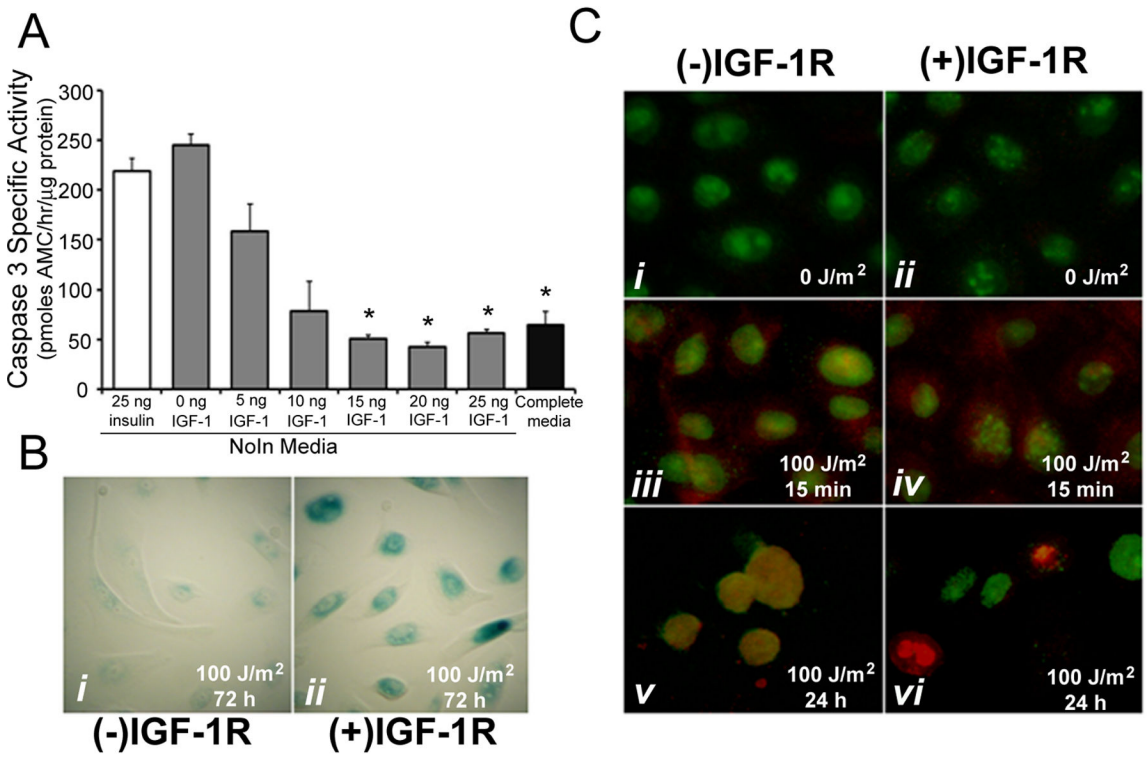

Figure 1. Exogenous IGF-1 (but not insulin) regulates the response of human keratinocytes to UVB irradiation

(A) When insulin is withheld from keratinocyte media, keratinocytes become more sensitive to UVB-induced apoptosis. Keratinocytes were grown in EpiLife NoIn media for 16 h. At that time the media was replaced with EpiLife Complete media, EpiLife NoIn media, or EpiLife NoIn media containing the indicated concentrations of IGF-1 or insulin for thirty min and then irradiated with 400 J/m^2 of UVB. Keratinocytes were harvested six hours post-irradiation and assayed for the induction of apoptosis by measuring the induction of caspase 3 specific activity. Error bars indicate the standard error of the mean; the asterisks indicate significant difference between caspase 3 specific activities derived from keratinocytes grown in unsupplemented EpiLife NoIn media (p<0.005, t-test). The data presented represent three independent assays. **(B)** Keratinocytes irradiated in the absence of IGF-1 do not undergo UVB-induced senescence. Keratinocytes were grown in EpiLife Complete or EpiLife NoIn media for 16 h and then irradiated with 100 J/m^2 of UVB. Twelve hours post-irradiation, the media on all of the plates was replaced with fresh EpiLife Complete media. Seventy-two hours post-irradiation, the keratinocytes were assayed for the expression of senescence-associated β-galactosidase activity (nuclei of senescent cells stain blue). **(C)** Keratinocytes lacking IGF-1R activation can replicate UVB-damaged DNA. Keratinocytes were grown in EpiLife NoIn (*i, iii, v*) or EpiLife Complete (*ii, iv, vi*) media for 16 h and then irradiated with 100 J/m^2 of UVB. At 0 (*i, ii*), 0.25 (*iii, iv*) or 24 (*v, vi*) h post-irradiation, the keratinocytes were fixed and assayed for the expression of Ki67 (appearing green) and the presence of UVB-induced thymidine dimers (appearing red). Cells expressing Ki67 and containing thymidine dimers appear yellow.

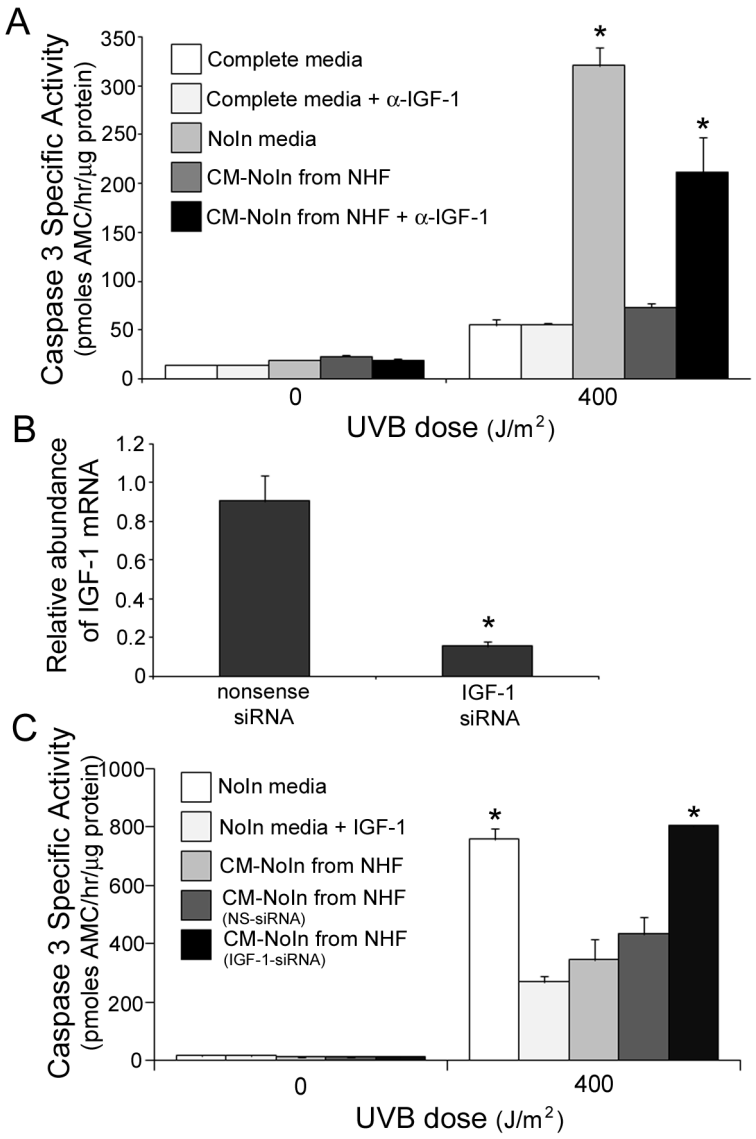

Figure 2. IGF-1 secreted by normal human fibroblasts can protect keratinocytes from UVB-induced apoptosis

(A) To determine if normal human fibroblasts can produce a protective factor which can activate the IGF-1R and restore the normal UVB response of keratinocytes, normal human fibroblasts were grown in the EpiLife NoIn media for 48 h (referred to as Conditioned Media, CM). Keratinocytes were grown in EpiLife NoIn media for 48 h and subsequently the media was replaced with EpiLife Complete media, EpiLife NoIn media, or EpiLife NoIn-CM derived from fibroblasts. Aliquots of EpiLife Complete media and CM-NoIn were treated with a neutralizing antibody to IGF-1 (Calbiochem, α-IGF-1, Ab2; inhibits 50% at suggested concentration) 15 min prior to adding it to the keratinocytes. One hour following media exchange, the keratinocytes were irradiated with 0 or 400 J/m^2 of UVB. Six hours post-irradiation, the keratinocytes were harvested and assayed for the induction of apoptosis. Error bars indicate the standard error of the mean; the asterisk indicates significant difference between caspase 3 specific activities derived from keratinocytes grown in

Complete media versus NoIn media ($p<0.01$, t-test). The data presented represent three independent assays. **(B and C)** Fibroblasts were treated with IGF-1-specific siRNA or non-specific siRNA for 24 h. NoIn media was then added to the fibroblasts for 48 h. At that time, the media was removed, filter-sterilized, and added to keratinocyte cultures. **(B)** qRT-PCR analysis of IGF-1 expression in fibroblasts following the indicated siRNA treatment. Error bars indicate the standard error of the mean; asterisk indicates significant ($p<0.02$, t-test) difference from nonsense siRNA-treated keratinocytes. **(C)** Keratinocytes were grown in NoIn media for 48 h, then the indicated media was added for 1 h. At that time, the keratinocytes were irradiated with the indicated dose of UVB and harvested six hours post-irradiation for caspase 3 analysis. Error bars indicate the standard error of the mean; the asterisks indicate significant difference between caspase 3 specific activities derived from keratinocytes grown in NoIn media + !GF-1 ($p<0.03$, t-test). The data presented represent three independent assays.

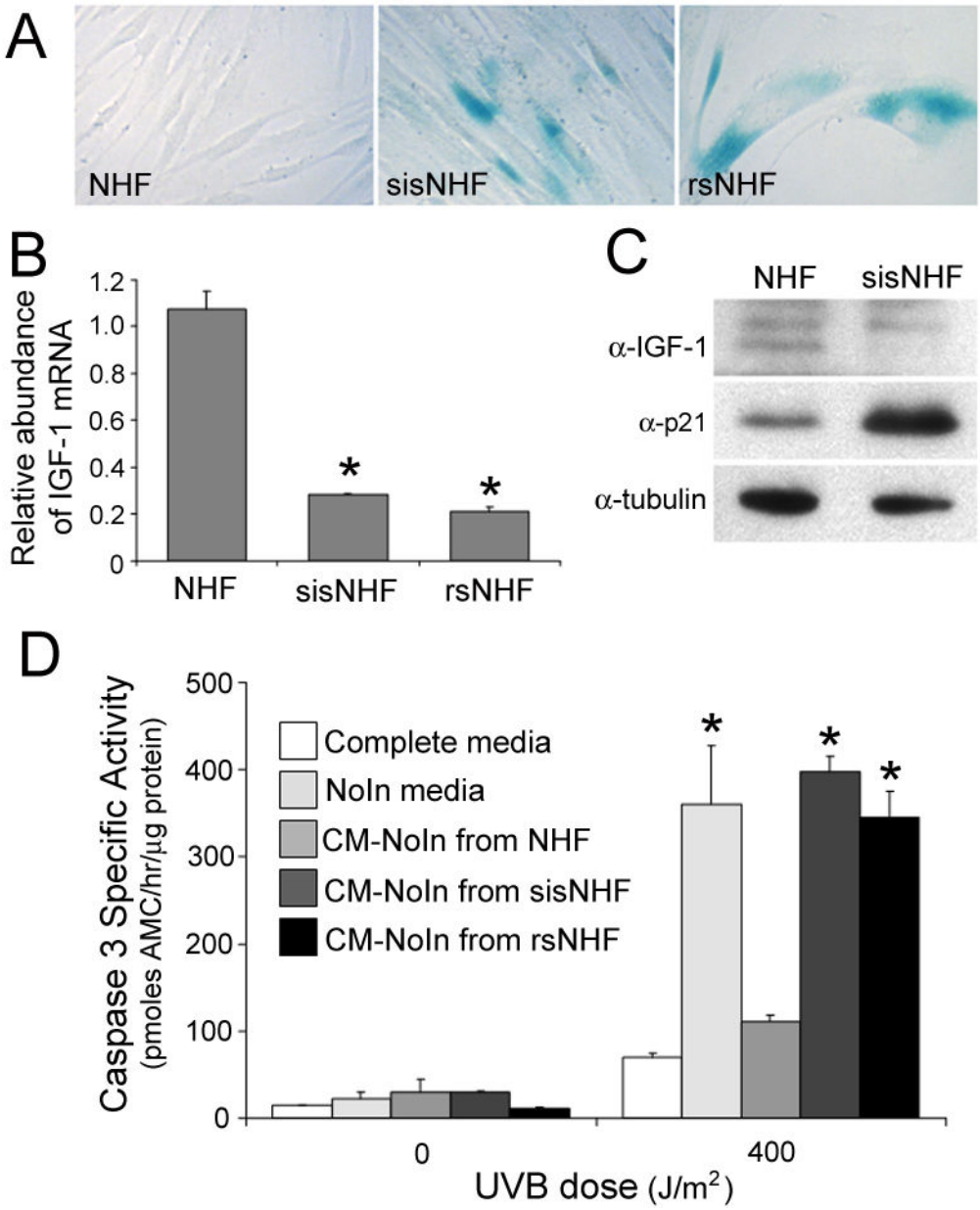

Figure 3. Induction of senescence (stress-induced or replicative) silences IGF-1 expression in fibroblasts and abolishes the protective effect of conditioned media on UVB-induced apoptosis in keratinocytes

Fibroblasts were treated with 600 μM H_2O_2 for 2 h and maintained in culture for 72 h (sisNHF) or serially passaged until they reached replicative senescence (rsNHF). **(A)** Fibroblasts stained for senescence-associated β-galactosidase activity (senescent cells are blue, appearing dark in the image) **(B)** qRT-PCR analysis of IGF-1 expression. Error bars indicate the standard error of the mean; asterisks indicate significant (p<0.03, t-test) difference from proliferative fibroblasts. **(C)** Proteins from the indicated cell lysates were immunoblotted and probed with the specified antibodies. **(D)** Conditioned media from proliferative fibroblasts (NHF-CM) but not from senescent fibroblasts (sisNHF CM or rsNHF-CM) protect keratinocytes from UVB-induced apoptosis. Error bars indicate the

standard error of the mean; the asterisks indicate significant difference between caspase 3 specific activities derived from keratinocytes grown in Complete media ($p<0.005$, t-test). The data presented represent three independent assays.

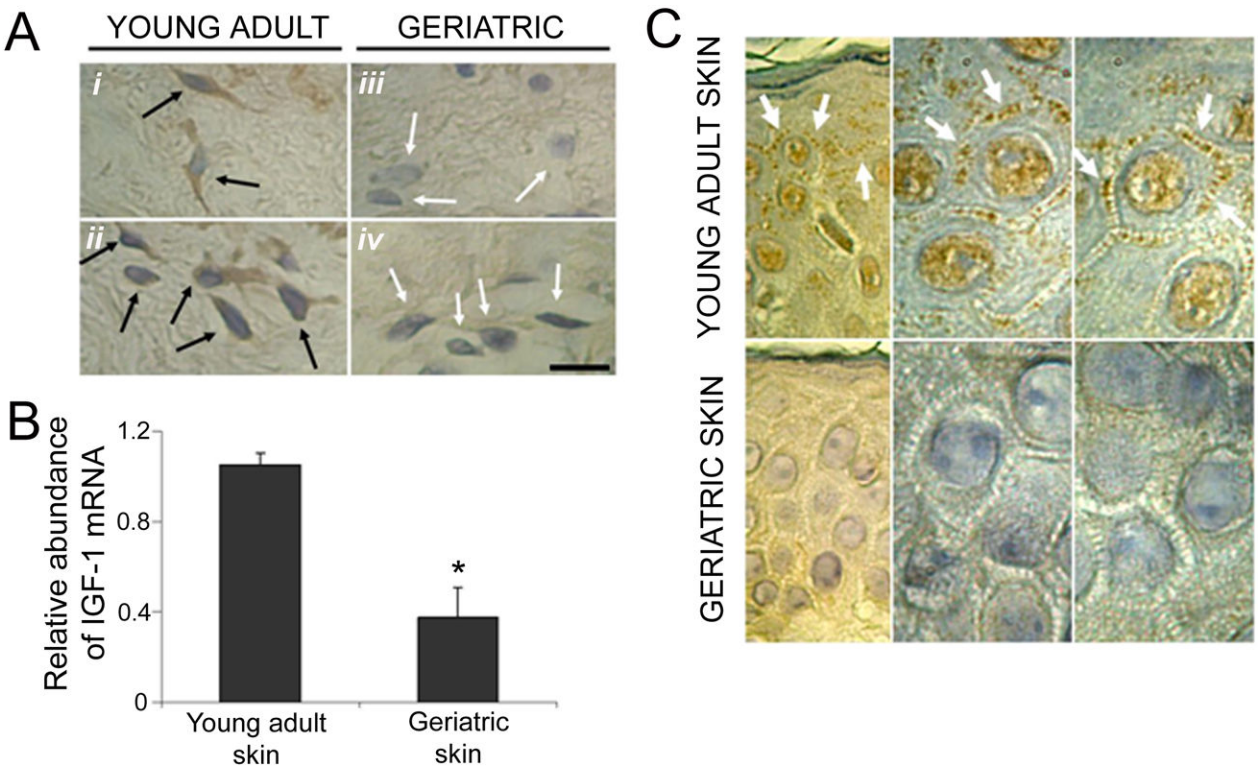

Figure 4. Decreased expression of IGF-1 in dermal fibroblasts and a corresponding decrease in IGF-1R activation in epidermal keratinocytes in geriatric skin

(A) Skin from sun-protected areas of young adult (20-28 years old, panels ***i, ii***) or geriatric (≥65 years old, panels ***iii, iv***) were stained with antibodies specific to human IGF-1 and visualized by anti-rabbit-HRP/DAB staining (positive staining indicated by brown precipitate). The slides were counterstained with hematoxylin. IGF-1 expression is readily observed in individual dermal fibroblasts from young skin (black arrows) but not in geriatric skin (white arrows). **(B)** Total RNA was extracted from biopsies of skin derived from young adult and geriatric skin. The relative amount of IGF-1 mRNA was determined by qRT-PCR analysis standardized to actin mRNA levels. Error bars indicate the standard error of the mean; asterisk indicates significant ($p<0.01$, t-test) difference between the IGF-1 mRNA levels from young adult skin versus geriatric skin. **(C)** Skin from sun-protected areas of young adult (20-28 years old, *top panels*) or geriatric (≥65 years old, *bottom panels*) were stained with antibodies specific to activated (tyrosine-phosphorylated) IGF-1R and visualized by anti-rabbit-HRP/DAB staining (positive staining indicated by brown precipitate). The slides were counterstained with hematoxylin. High power micrographs indicate activated IGF-1R expression in nuclei and in discrete pockets at the cell surface (examples indicated by white arrows) of individual epidermal keratinocytes in young skin but not in geriatric skin.

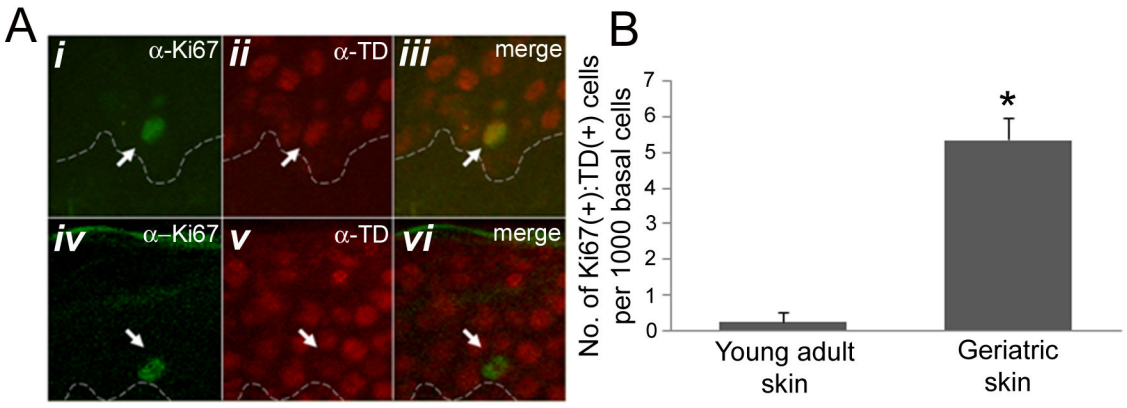

Figure 5. Deficient IGF-1 expression in geriatric fibroblasts increases mutagenic potential in keratinocytes following UVB irradiation

(A) Example of UVB-response assay. A 2 cm^2 area on the lower back of a 75 year old volunteer was irradiated with UVB (350 J/m^2). Twenty-four hours following irradiation, a four mm punch biopsy was obtained from the irradiated skin. Sections of formalin-fixed, paraffin-embedded tissue were stained with both α-Ki67 and α-thymidine dimer antibodies (panels ***i, iv***: α-Ki67 immunofluorescence only (staining green); panels ***ii, v***: α-thymidine dimer immunofluorescence only (staining red); panels ***iii, vi***: α-Ki67 and α-thymidine dimer double-immunofluorescence (double-positive cells staining yellow). Arrows in ***i, ii, iii*** and ***iv, v, vi*** indicate the location of the same cell(s) in each of the respective panels. The top series of panels is an example of a cell positive for both Ki67 and thymidine dimmers (staining yellow, inappropriate UVB response) while the cell indicated in bottom panels is only positive for Ki67 (staining green, appropriate UVB response). The location of the basement membrane is indicated by a grey dashed line. **(B)** Geriatric keratinocytes respond inappropriately to UVB irradiation. A 2 cm^2 area on the lower back of young adult (20-28 years old; YA) or geriatric (> 65 years old; GA) volunteers were irradiated with UVB (350 J/m^2). Twenty-four hours following irradiation, a four mm punch biopsy was obtained from the irradiated skin and a three mm punch biopsy was removed from unirradiated skin. The tissues were processed as described in **(A)**. The number of basal layer keratinocytes positive for only Ki67, positive for only TD, or positive for both Ki67 and TD were determined for the entire length of each biopsy. Similar sections were stained with H&E to determine the total number of basal layer keratinocytes in each biopsy. The graph represents the number of replicating keratinocytes in the UVB-irradiated skin that contains UVB-induced DNA damage per 1000 basal layer cells. The results presented were derived from six young adult and six geriatric volunteers. Asterisk indicates a statistically significant difference between the two cohorts ($p<0.0001$, student t-test).

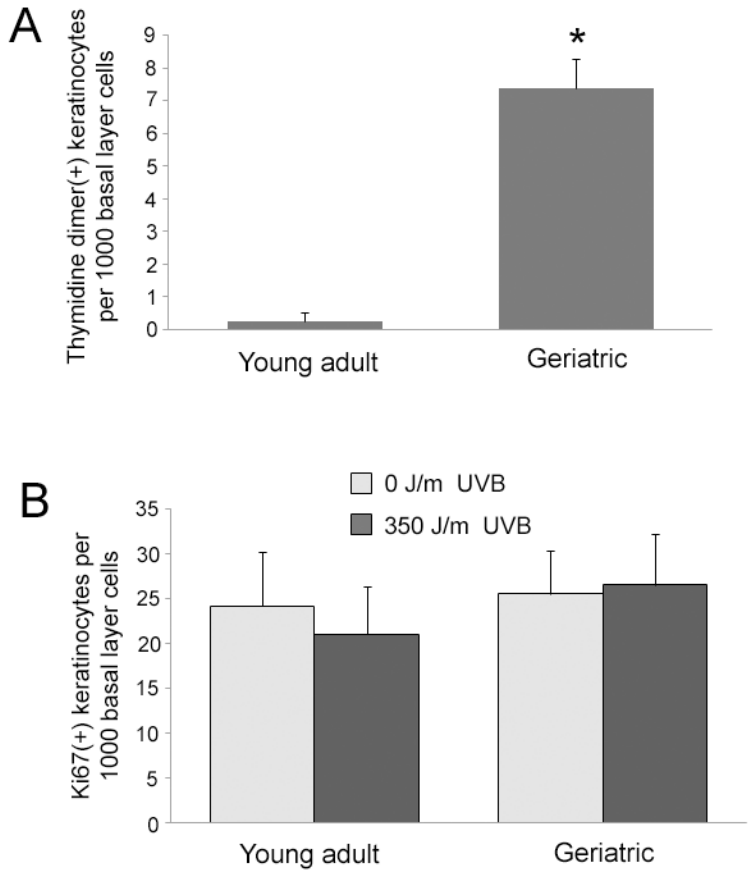

Figure 6. Young adult keratinocytes repair UVB-induced DNA damage more quickly than geriatric keratinocytes

(A) Experiment was conducted as described in Figure 5. The graph represents the total number of keratinocytes in the UVB-irradiated skin that contains UVB-induced DNA damage per 1000 basal layer cells from each treatment group. The results presented were derived from six young adult and seven geriatric volunteers. Asterisks indicates a statistically significant difference between the young adult and geriatric cohorts ($p<0.0001$, student t-test). **(B)** No difference between the number of replicating keratinocytes in young adult or geriatric skin. Experiment was conducted as described in Figure 5. The graph represents the total number of replicating keratinocytes per 1000 basal layer cells from each treatment group. There was no statistically significant difference between and of the treatment groups.

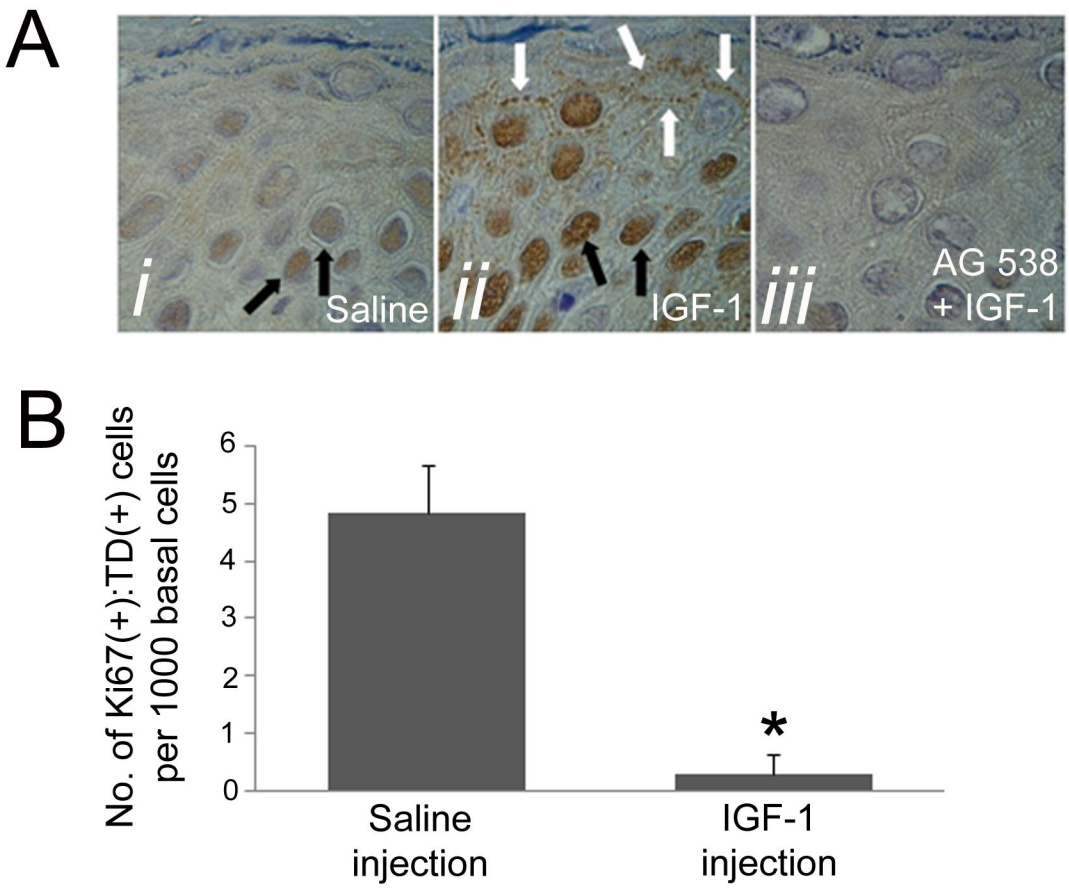

Figure 7. Exogenous IGF-1 restores appropriate UVB-response to geriatric skin

(A) Intradermal injection of IGF-1 activates the epidermal IGF-1R. In vitro explants of full-thickness human skin were analyzed for the activation of the IGF-1R using an antibody specific for tyrosine-phosphorylated IGF-1R. ***i***.) Control untreated explanted skin; ***ii***.) IGF-1 (200 ng in 50 μl of saline) was injected into the dermis of explanted human skin 30 minutes prior to harvesting the skin; ***iii***.) AG 538 (a specific small molecule inhibitor or the IGF-1R) was topically applied to the explanted human skin for 30 min prior to sub-dermal injection of IGF-1 (as described in ***ii***). White arrows indicate brown punctate cell membrane staining and black arrows indicate positively-staining brown nuclei. **(B)** Exogenous IGF-1 corrects the inappropriate UVB-response in geriatric skin. Isolated areas on the lower backs of geriatric volunteers were injected with 50 μl of saline and 50 μl of saline containing 200 ng of IGF-1 just beneath the epidermis. Thirty minutes following the injections, each site was irradiated with 350 J/m^2 of UVB. At twenty-four hours post-irradiation, biopsies were obtained from each irradiated site and from an unirradiated site. The tissues were processed as described in (Figure 5b). The results presented in the graph were derived from seven geriatric volunteers. Asterisk indicates a statistically significant difference between the saline and IGF-1 treated sites (p<0.0001; student t-test).

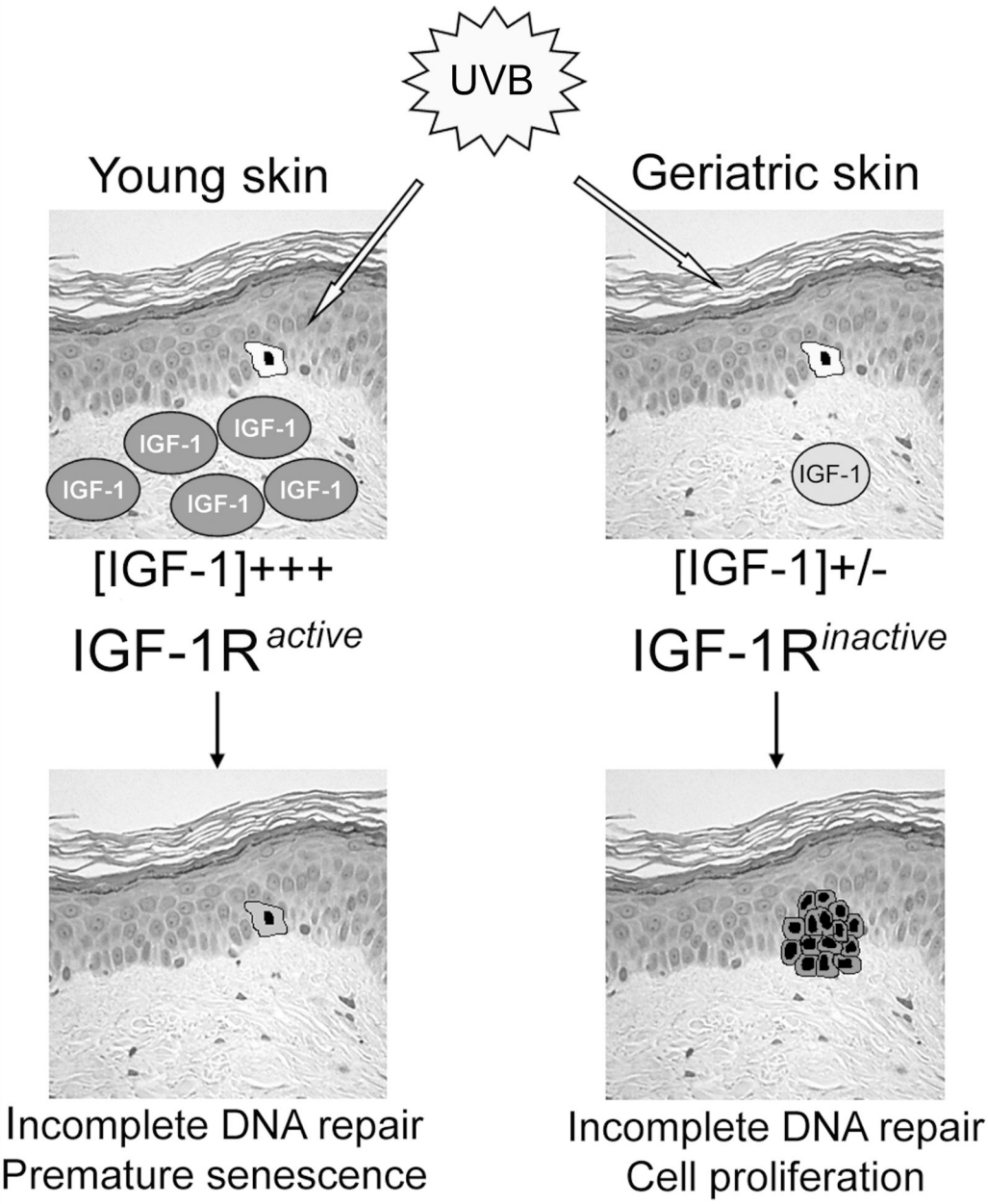

Figure 8. The influence of aging on IGF-1 expression in the skin and its role in UVB-induced carcinogenesis

Keratinocytes in aged epidermis exposed to UVB wavelengths in sunlight may respond inappropriately to the UVB exposure. The dermis of young adults produces sufficient levels of IGF-1 to activate the IGF-1R on epidermal keratinocytes. The appropriate activation of the IGF-1R on keratinocytes leads to the induction of stress-induced senescence following sufficient UVB exposure. In contrast, the expression of IGF-1 is silenced in aged dermis. The consequence of diminished IGF-1 expression is a lack of IGF-1R activation in epidermal keratinocytes. Instead of undergoing stress-induced senescence, the aged keratinocytes are able to proliferate in the presence of UVB-damaged DNA. We hypothesize

this decrease in IGF-1 expression with advancing age is a contributor to the increase in non-melanoma skin cancer seen in geriatric patients.